RESEARCH LETTER

Nitrogen source influences natural abundance $^{15}$N of Escherichia coli

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Abstract

Escherichia coli cells were forced to mineralize or assimilate nitrogen in vitro by manipulating substrate carbon and nitrogen availability. When grown on an organic nitrogen source, E. coli cells released $\text{NH}_4^+$ and were enriched in $^{15}$N relative to the nitrogen source (1.6–3.1%). However, when cells were grown on an inorganic nitrogen source, the biomass was depleted (6.1–9.1%) relative to the source. By measuring $^{15}$N enrichment of microorganisms relative to nitrogen pools, ecosystem ecologists may be able to determine if microorganisms are assimilating or mineralizing nitrogen.

Introduction

Natural abundance stable isotopes are effective tools for elucidating biogeochemical processes in microbial ecology (Högberg, 1997; Robinson, 2001; Boschker & Middelburg, 2002; Staddon, 2004). Exploiting differences in isotope signatures between nutrient source and sink has made it possible to link microbial activity to ecosystem processes (Miyazaki et al., 1980; Orphan et al., 2001; Townsend et al., 2003). Ecosystem ecologists are increasingly interested in measuring the natural abundance isotopic composition of the microbial biomass or DNA extracted from the environment in order to interpret dominant microbial processes (Dijksta et al., 2006a, b; Schwartz et al., 2007). However, mixing of nitrogen (N) source pools and the great number of isotopically fractionating element transformations in the environment creates difficulty in interpreting environmental isotopic signatures (Högberg, 1997; Robinson, 2001). For this reason, researchers have called for controlled culture experiments to clarify mechanisms of cellular enrichment (Gannes et al., 1997; Hobbie et al., 2001). Past work shows that microbial cells can be either enriched or depleted in $^{15}$N, with no clear consensus on the mechanism determining the outcome. For example, Macko & Estep (1984) showed that V. harveyi cells cultured on different amino acids are either enriched or depleted in $^{15}$N. Henn & Chapela (2004) showed that ammonia (NH$_3$) assimilation in fungi led to strong depletion of $^{15}$N in the microbial biomass relative to the nitrogen source. Hobbie et al. (2001) have shown that $^{15}$N natural abundance measurements can differentiate between organisms of different trophic strategies. To date there are no reports comparing $^{15}$N enrichment of bacteria grown in media with inorganic vs. organic nitrogen sources.

In environments with low carbon (C) availability, organisms utilize organic nitrogen-substrates such as amino acids or proteins as a source of carbon. Following deamination of nitrogenous carbon compounds, $\text{NH}_4^+$ is released from the cell (Paul & Clark, 1996). Because transport of $\text{NH}_3$ is a fractionating process that strongly selects for the lighter nitrogen isotope, bacteria forced to mineralize nitrogen may become enriched in $^{15}$N while cells that assimilate $\text{NH}_3$ will become depleted in $^{15}$N (Henn & Chapela, 2004; Dijkstra et al., 2006a, b). If true, this observation would be of great
use to ecosystem ecologists because microbial assimilation and mineralization of nitrogen strongly influence nitrogen availability to plants, and thus plant growth.

We cultured *E. coli* in defined media where the nitrogen source and concentration were controlled. Nutrient availability in the media was manipulated to force organisms to either mineralize or assimilate nitrogen. To test if cells become enriched in $^{15}$N relative to the nitrogen source when releasing NH$_4^+$ and depleted in $^{15}$N relative to source when assimilating NH$_4^+$, we measured the $\delta^{15}$N of the *E. coli* cells among these treatments.

**Materials and methods**

Stock *E. coli* strain DH5α-T1 (Invitrogen Corp., Carlsbad, CA) was inoculated (c. $10^6$ cells) into flasks with 250 mL of minimal medium made with either (NH$_4$)$_2$SO$_4$ or glycine as the sole nitrogen source. Cultures were incubated at 37°C with shaking. Carbon and nitrogen in media were added to yield C:N molar ratio of 5, 15, 20, 25, and 50 for each nitrogen source. All media contained 7 g L$^{-1}$ Na$_2$HPO$_4$, 2 g L$^{-1}$ KH$_2$PO$_4$, 0.1 g L$^{-1}$ MgSO$_4$, 0.02 g L$^{-1}$ CaCl$_2$, 2 H$_2$O, 0.5 g L$^{-1}$ glycine, 2.52 g L$^{-1}$ glucose, 0.02 g L$^{-1}$ ATCC Trace Element Supplement, and 10 mL L$^{-1}$ ATCC Vitamin Supplement, and were adjusted to pH 7.5. The media were filter sterilized using a 0.2 μm Nalgene vacuum filter. Ammonium/glucose medium with C:N ratios of 5, 15, 20, 25, and 50 contained 1 g L$^{-1}$ (NH$_4$)$_2$SO$_4$ and 2.5 g L$^{-1}$ d(+)-glucose, 0.5 g L$^{-1}$ (NH$_4$)$_2$SO$_4$ and 3.75 g L$^{-1}$ d(+)-glucose, 0.5 g L$^{-1}$ (NH$_4$)$_2$SO$_4$ and 5.0 g L$^{-1}$ d(+)-glucose, 0.25 g L$^{-1}$ (NH$_4$)$_2$SO$_4$ and 3.12 g L$^{-1}$ d(+)-glucose, and 0.20 g L$^{-1}$ (NH$_4$)$_2$SO$_4$ and 5.0 g L$^{-1}$ d(+)-glucose, respectively. Glycine/glucose medium with C:N ratios of 5, 15, 20, 25, and 50 contained 1.4 g L$^{-1}$ glycine and 1.5 g L$^{-1}$ d(+)-glucose, 0.5 g L$^{-1}$ glycine and 2.86 g L$^{-1}$ d(+)-glucose, 0.5 g L$^{-1}$ glycine and 3.96 g L$^{-1}$ d(+)-glucose, 0.25 g L$^{-1}$ glycine and 2.52 g L$^{-1}$ d(+)-glucose, and 0.20 g L$^{-1}$ glycine and 4.22 g L$^{-1}$ d(+)-glucose, respectively.

Each treatment had a total of three replicates. At five points along the growth curve, subsamples were removed from each flask. *Escherichia coli* growth was determined through absorbance measurements at 600 nm with a spectrophotometer (Fig. 1) (Eppendorf North America Inc., Westbury, NY). At all harvest times the concentration of cells was much higher than the 4000 cells mL$^{-1}$ added to the medium at the beginning of the incubation so that the isotopic signature of the inoculant did not impact the isotopic composition of the cells at harvest. Cells were separated from media by centrifugation and were washed twice with 0.085% (w/v) NaCl. Media and cells were maintained at −20°C before analysis. Cells were dried under laminar flow for 24 h, or until completely dry and ground into a fine powder by hand with a mortar and pestle and packed into 3.5 mm × 9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA). Ammonium concentration in media was determined by flow injection analysis on a Lachat QuickChem 8000 Automated Ion Analyzer (Lachat Instruments, Loveland, CO) (Prokopy, 2003).

To measure $\delta^{15}$N of NH$_4^+$, acid–base diffusion was used to prepare NH$_4^+$ samples for $\delta^{15}$N analysis (Stark & Hart, 1996). The volume of sample required per diffusion was targeted for 40 μg of nitrogen. National Institute of Standards and Technology (NIST) standard reference materials NBS-1 and NBS-2, and (NH$_4$)$_2$SO$_4$ were also diffused using this procedure. For each sample, a 7-mm diameter Whatman GF/F filter disc was acidified with 20 μL of 2.5 M KHSO$_4$ and sandwiched between two layers of Teflon tape (Stark & Hart, 1996). This acid trap package was added to media and 0.5 M NaCl solution. The solution was saturated with MgO and sealed, forcing the NH$_4^+$ in the solution to NH$_3$, which volatilized and collected on the acid trap as NH$_4^+$. Samples were incubated at 30°C for 7 days to maximize recovery of NH$_4^+$ and NH$_3$ (Sigman et al., 1997; Holmes et al., 1998). Acid traps and filters were dried in a desiccator equipped with an acid trap to avoid contamination by atmospheric NH$_3$. Once dry, filters were removed from the Teflon tape and packed into 5 mm × 9 mm tin capsules (Costech). Samples and diffused standards were analyzed on the mass spectrometer within 48 h, or were stored on desiccant to avoid disintegration of the tin from KHSO$_4$. 

![Fig. 1. Changes in OD of *Escherichia coli* cultures grown in (a) NH$_4^+$/glucose media or (b) glycine/glucose over time at different initial C : N ratios of the media.](http://femsle.oxfordjournals.org/content/282/4/246/F1.large.jpg)
The natural abundances of $^{15}$N of cells and of glass filters were measured using a Carlo Erba NC 2100 Elemental Analyzer (CE Instruments, Milan, Italy) interfaced with a Thermo-Finnigan Delta Plus XL isotope ratio mass spectrometer (Thermo-Electron Corp., Bremen, Germany). The $^{15}$N compositions were expressed in standard delta notation ($\delta^{15}$N) in parts per thousand ($\permil$) relative to atmospheric N$_2$, where $\delta = 1000 \times [(R_{\text{sample}}/R_{\text{standard}}) - 1]$ and $R$ is the molar ratio $^{15}$N$/^{14}$N (Lajtha & Michener, 1994). The external precision on repeated measurements of an internal standard (NIST 1547 – peach leaves) was < 0.20$. The SD of standards prepared by diffusion was 0.8$ or better.

Repeated measures multivariate ANOVA (MANOVA) test was used to determine if isotopic composition of microbial cells were significantly different between treatments. A separate MANOVA was run for each nitrogen source. The response variables, time, C : N ratio, and the time by C : N interaction, were determined in cultures of E. coli. All statistical analyses were performed using JMP 4 statistical package (SAS Institute, Cary, NC). Alpha values < 0.05 were considered statistically significant in these analyses.

Results and discussion

The aim of this study was to test if the nitrogen isotopic composition of E. coli was impacted by growth on an inorganic vs. organic nitrogen source and to determine whether the relative availability of carbon and nitrogen influenced the degree of $^{15}$N enrichment.

**Fig. 2.** Changes in $\delta^{15}$N of Escherichia coli cells grown in (a) NH$_4^+$/glucose media or (b) glycine/glucose over time at different initial C : N ratios of the media. Symbols represent mean values and error bars represent ± 1 SE. For samples without error bars, the SE was smaller than the symbol. Dotted line represents $\delta^{15}$N of (a) NH$_4^+$ or (b) glycine used to make media.

**Fig. 3.** Changes in NH$_4^+$ concentrations in (a) NH$_4^+$/glucose or (b) glycine/glucose media with different initial C : N. Symbols represent mean values and error bars represent ± 1 SE. For samples without error bars, the SE was smaller than the symbol.
diminishes as more of the source pool of nitrogen is utilized (Robinson, 2001). In treatments with C:N ratios of 15, 20, 25, and 50, the δ15N of microbial biomass increased over time and became enriched (1.9–3.2‰) relative to the source nitrogen. Carbon starvation may have forced these cells to metabolize their own proteins and other biomolecules as a source of carbon and energy, resulting in nitrogen mineralization. Time, medium C:N ratio, and the interaction between these factors significantly affected the δ15N content of microbial biomass when grown in inorganic nitrogen (F = 8.06, P < 0.01; F = 994.87, P < 0.01; F = 16.24, P < 0.01, respectively). In the NH4+/glucose media, NH4+ concentrations declined over time, showing cells were taking up and assimilating NH4+ (Fig. 3a). When NH4+ was offered as a nitrogen source, cells assimilated nitrogen regardless of the substrate C:N ratio, as demonstrated by the rapid increase in NH4+ concentrations in the media. NH4+ was eventually exhausted in media of all C:N treatments except for the medium with a C:N ratio of 5, where NH4+ remained in high concentrations.

When E. coli was cultured in NH4+/glucose medium with a C:N ratio of 5, the δ15N of NH4+ increased over time from 1.9‰ to 6.9‰ (Fig. 4a) showing that E. coli preferentially assimilated the light isotope of nitrogen, leaving heavier NH4+ behind. This pattern is representative of closed, unidirectional systems in which isotopic fractionation follows Raleigh kinetics (Evans, 2001; Robinson, 2001). We observed a consistent isotope separation between cells and ammonium of 10.1‰ (± 0.3). In a study of ammonium assimilation by marine bacterial assemblages, Hoch et al. (1994) estimated isotopic fractionation factors to range between 5‰ and 20‰. The pH remained below 8 in all experiments, well below the pH at which the equilibrium between NH3 and NH4+ favors volatilization of isotopically depleted NH3 (Bigeleisen, 1965). Therefore, it is unlikely that pH influenced the isotopic composition of microorganisms or nitrogen pools. The δ15N of NH4+ in media with C:N ratios of 15, 20, 25, and 50 could not be measured due to low NH4+ concentrations in these treatments.

Escherichia coli cells grown in glycine/glucose media were initially enriched in 15N (1.6–3.1‰) relative to the nitrogen source (Fig. 2b). In the first 50 h of growth, cells in media with a C:N of 5 had higher δ15N enrichment values than C:N treatments 15, 20, 25, and 50. Exudation of 14NH3 has been suggested as a mechanism of microbial 15N enrichment when cells utilize organic nitrogen-substrates as a carbon source (Dijkstra et al., 2006b). The cells in the C:N 5 treatment metabolized all the glucose in the media and were forced to utilize nitrogenous substrates as a source of carbon before the other treatments, as indicated by higher ammonium concentrations in the medium (Fig. 3b). This probably led to higher microbial enrichment in 15N. However, over time E. coli cells in all C:N treatments had δ15N values that were similar to the initial signature of glycine. Like cultivation in NH4+/glucose media, time, medium C:N, and the interaction of these factors significantly affected the δ15N of microbial cells across all C:N treatments when grown on organic nitrogen (F = 16.32, P < 0.01; F = 51.49, P < 0.01; F = 2.21, P = 0.05, respectively).

In the glycine/glucose media, the NH4+ concentration increased over time in all C:N treatments, indicating cells were secreting NH4+ (Fig. 3b). Over time, media with C:N ratios higher than 5 had very little remaining NH4+, indicating the released NH4+ was assimilated by E. coli. However, mineralization of glycine remained high in the C:N 5 treatment, demonstrating cells remained carbon limited.

Isotopic composition of NH4+ in glycine/glucose medium with a C:N ratio of 5 was similar to the δ15N of glycine after 75 h of growth (Fig. 4b). Before this, the NH4+ concentration in the medium was too low to measure δ15N of NH4+. At all time points in C:N treatments 15, 20, 25, and 50, the δ15N of NH4+ could not be measured because NH4+ concentrations were too low to obtain sufficient nitrogen for analysis.

Both NH4+ assimilation and nitrogen mineralization require NH3 to be transported across the plasma membrane of microorganisms. Our results suggest that this process fractionates nitrogen isotopes, favoring 14N uptake into the cell cytoplasm over 15N. Consequently, microorganisms become enriched in 15N when secreting NH4+ and depleted.
in $^{15}$N when importing NH$_4^+$]. Alternatively, enzymatic reactions inside the cell such as deamination or transamination of specific amino acids [Hoch et al., 1994] may explain the differences in isotopic fractionation when cells are assimilating versus mineralizing nitrogen. These results can be used to interpret $^{15}$N isotopic patterns of microbial communities in the environment. We predict that microorganisms will be enriched in $^{15}$N relative to the source nitrogen in carbon limited environments where nitrogen substrates are important sources of carbon and nitrogen mineralization rates are high. Conversely, in environments where large amounts of carbon and NH$_4^+$ are available, microorganisms will assimilate nitrogen and consequently be depleted in $^{15}$N relative to their nitrogen source. The degree to which microorganisms will be depleted in $^{15}$N relative to their nitrogen source in environmental samples could be very small if microbial uptake causes the concentrations of NH$_4^+$ to be so low that fractionation barely occurs.

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**References**